

Spectral and Chemical Characterization of Phosphates Associated with Humic Substances

Zhongqi He,* Tsutomu Ohno, Barbara J. Cade-Menun, M. Susan Erich, and C. Wayne Honeycutt

ABSTRACT

Because humic substances are involved in many processes in soils and natural waters, characterization of phosphorus (P) associated with humic substances may shed light on the function of natural organic matter in P cycling and nutrition. In this study, we investigated the spectral features and potential availability of P in the International Humic Substance Society (IHSS) Elliott Soil humic acid standard (EHa), Elliott soil fulvic acid standard II (EFa), Waskish peat humic acid reference (WHa), and Waskish peat fulvic acid reference (WFa) by fluorescence spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), solution ^{31}P nuclear magnetic resonance (NMR), 3-phytase incubation, and UV irradiation. We observed more similar spectral features between EHa and EFa as well as between WHa and WFa than between the two humic acids or two fulvic acids themselves. Phosphorus in WHa and WFa was mainly present in the orthophosphate form. However, only about 5% was water soluble. After treatment by both UV irradiation and enzymatic hydrolysis, soluble orthophosphate increased to 17% of the P in WHa, and 10% of the P in WFa. Thus, it appears that a large portion of P in Waskish peat humic substances was not labile for plant uptake. On the other hand, both orthophosphate and organic phosphate were present in EHa and EFa. Treatment by both UV irradiation and enzymatic hydrolysis increased soluble orthophosphate to 67% of the P in EHa and 52% of the P in EFa, indicating that more P in Elliott soil humic substances was potentially bioavailable. Our results demonstrated that source (soil vs. peat) was a more important factor than organic matter fraction (humic acid vs. fulvic acid) with respect to the forms and lability of P in these humic substances. This work represents a much more complete characterization of humic substance-bound P than previously reported in the literature, thus providing a comprehensive approach for improved understanding of P cycling in relation to ecosystem function.

HUMIC SUBSTANCES are involved in many processes in soils and natural waters: for example, soil weathering, plant nutrient bioavailability, pH buffering, trace metal mobility and toxicity, and degradation and transport of hydrophobic organic chemicals (Stevenson, 1982a). Phosphorus has long been known to be present in natural organic matter (NOM) from various sources, and found mainly in the humic fractions (Stevenson, 1982b and references therein). Even though P may not be incorporated into the molecular structure of humic substances, the high affinity of NOM for Al and Fe can bind P to produce organic matter-metal-P complexes (Tan, 2003). Labora-

tory and field experiments have shown that mobile humic substances may mediate P transport and result in P leaching in forest soils (Donald et al., 1993; Jones et al., 1993; Schoenau and Bettany, 1987). Similarly, Hens and Merckx (2001) proposed that the interaction among metals, organic matter, and P controls the dynamics of mobile colloidal P species in excessively fertilized sandy soils. Although these studies provide valuable insight into the function of NOM in P cycling, the identity and stability of P associated with humic substances are not fully understood.

Numerous spectral and chemical approaches can be used to characterize the P associated with NOM. Fourier-transform infrared and ^{13}C NMR spectroscopies have been used to identify functional groups and their structural arrangements in NOM (Bedrock et al., 1995; Brannon and Sommers, 1985; Fan et al., 2000; Giusquiani et al., 1998; Olk et al., 2000; Wang and Xing, 2005). Fluorescence spectroscopy has been applied recently in studying NOM chemistry (Olk et al., 2000; Merrit and Erich, 2003; Wu et al., 2004; Sierra et al., 2005; Cory and McKnight, 2005), while solution ^{31}P NMR spectroscopy is a powerful tool for identifying the forms of P in the NOM and other environmental samples (Bedrock et al., 1995; Cade-Menun et al., 2002; Hawkes et al., 1984; Mahieu et al., 2000; Pant et al., 1999). Phosphatase hydrolysis can provide an estimate of hydrolyzable (bioavailable) organic P in environmental samples (Hayes et al., 2000; He et al., 2003, 2004; Hens and Merckx, 2001; Herbes et al., 1975; Pant and Warman, 2000; Shand and Smith, 1997), and abiotic instability of P associated with NOM may be evaluated by ultraviolet (UV) irradiation (Cotner and Heath, 1990; De Haan, 1993; Francko and Heath, 1982; Hens and Merckx, 2001). Whereas most attempts to characterize P associated with NOM have adopted one or two of these approaches to address a specific scientific question, efforts to characterize the P associated with NOM comprehensively using all these approaches have not been reported.

To promote critical comparisons of their experimental results on humic substances by researchers throughout the world, the IHSS (IHSS, 2005) has collected standard and reference humic substances. Unique features of the collection are: (i) it is accessible to researchers worldwide; (ii) all materials originated from carefully chosen and specified locations; (iii) all samples have been isolated by carefully controlled and supervised procedures that are fully documented; and (iv) all materials have been thoroughly homogenized. Thus, as the first step in

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Abbreviations: EFa, IHSS Elliott soil standard fulvic acid II; EHa, IHSS Elliott soil standard humic acid; EEM, emission-excitation matrix; EM, emission; EX, excitation; FT-IR, Fourier-transform infrared spectroscopy; IHSS, the International Humic Substances Society; LHa, IHSS Leonardite standard humic acid; NMR, nuclear magnetic resonance spectroscopy; NOM, natural organic matter; PARAFAC, parallel factor analysis; PHa, IHSS Pahokee peat reference humic acid; P_i , soluble inorganic orthophosphate determined by a molybdenum blue method; WFa, IHSS Waskish peat reference fulvic acid; WHa, IHSS Waskish peat reference humic acid.

our efforts to better understand the chemistry of P associated with humic substances, we used the spectral and chemical approaches mentioned above to investigate the forms and lability (potential bioavailability) of P in the IHSS Elliott soil humic acid standard, Elliott soil fulvic acid standard II, Waskish peat humic acid reference, and Waskish peat fulvic acid reference.

MATERIALS AND METHODS

Humic Substances

Six humic substances from three solid-phase source materials (soil, peat, and leonardite) were obtained from the International Humic Substances Society (IHSS, 2005): 1. Elliott soil standard humic acid 1S102H (EHa); 2. Elliott soil standard fulvic acid II 2S102F (EFa); 3. Waskish peat reference humic acid 1R107H (WHa); 4. Waskish peat reference fulvic acid 1R107F (WFa); 5. Leonardite standard humic acid 1S104H (LHa); and 6. Pahokee peat reference humic acid 1R103H (PHa). The Elliott soil is typical of the fertile prairie soils of the U.S. states of Indiana, Illinois, and Iowa. The IHSS sample was obtained from an undisturbed area on the grounds of the Joliet Army Ammunition Plant near Joliet, IL. The IHSS Waskish peat was collected in Pine Island Bog in Koochiching County, Minnesota. This is a Sphagnum bog peat typical of northern temperate regions. The Waskish series consists of deep, very poorly drained organic soils that formed mostly in slightly decomposed material from Sphagnum moss on raised bogs on glaciated terrain. Leonardite is produced by the natural oxidation of exposed lignite, a low-grade coal. The IHSS sample was obtained from the Gascoyne Mine in Bowman County, North Dakota, USA. The Pahokee peat is a typical agricultural peat soil of the Florida Everglades. The IHSS sample was obtained from the University of Florida Belle Glade Research Station. Large quantities of the bulk source materials were air-dried and then sieved to remove pebbles and gross fibrous matter. After they were homogenized, a sufficient quantity of each source material was set aside for isolation of the standard humic and fulvic acids. The IHSS used a hybrid procedure (Swift, 1996) to isolate humic and fulvic acids from these solid materials.

The first four humic substances listed above, which P contents were $>1 \text{ g kg}^{-1}$ of humic substance (Table 1), were used for full characterization work. Stock solutions of these four humic substances were made in 0.1 M NaOH based on 20 mg P L^{-1} stock solution rather than a same concentration of total humic substance as this work was focused on P in these substances.

Fluorescence Measurements

Fluorescence measurements of humic substances (3.3 mg humic substance L^{-1}) in 100 mM acetic acid/sodium acetate

buffer (pH 5.0, refer to as acetate buffer hereinafter) were obtained using a Hitachi F-4500 spectrofluorometer (Hitachi High Technologies America, Inc, San Jose, CA. Instrumental parameters were excitation (EX) and emission (EM) slits, 5 nm ; response time, 8 s ; and scan speed 240 nm min^{-1} . The excitation-emission matrix (EEM) fluorescence landscape was obtained by setting the EX range from 240 to 400 nm and EM range from 300 to 500 nm in 3-nm increments. Subtraction of a deionized-water blank EEM from each sample EEM was used to remove Raman scatter lines from the spectra. The Rayleigh scatter lines were removed by replacing the fluorescence intensity values with missing values in the region immediately adjacent to the region where $\text{EM} = \text{EX}$ and 2 EX . The EEM had a triangular shaped region where EM wavelength was less than the EX wavelength. These physically impossible data positions were set to zero.

The PARAFAC model can provide a chemically meaningful model of EEM fluorescence spectra (Smilde et al., 2004). For one fluorophore, the emission intensity at a specific wavelength, j , when excited at wavelength k , can ideally be approximated:

$$x_{jk} = ab_j c_k \quad [1]$$

where x_{jk} is the intensity of the light emitted at emission wavelength j at excitation wavelength k , a is the concentration (in an arbitrary scale) of the analyte, b_j is the relative emission emitted at wavelength j , and c_k is the relative amount of light absorbed at the excitation wavelength k . If several analytes are present then the intensity can be written as a function of these F analytes by simply summing the individual contributions:

$$x_{jk} = \sum_{f=1}^F a_f b_{jf} c_{kf} \quad [2]$$

In the above equation, the relative emission of analyte f at emission j is b_{jf} and the relative absorption at excitation k is c_{kf} , and the concentration of analyte f is a_f . Equation [2] implies that the contribution to the emission from each analyte is independent of the contributions of the remaining analytes. For several samples, and a_{if} being the concentration of the f th analyte in the i th sample, the model becomes:

$$x_{ijk} = \sum_{f=1}^F a_{if} b_{jf} c_{kf}, i = 1, \dots, I; j = 1, \dots, J; k = 1, \dots, K \quad [3]$$

This model of several samples is exactly the same as the PARAFAC model of a three-way array with typical elements x_{ijk} and hence the parameters a_{if} , b_{jf} , and c_{kf} can be determined by fitting a PARAFAC model to the three-way data equaling the set of EEMs. Using the correct number of components F , PARAFAC therefore directly provides estimates of the relative concentrations and excitation and emission spectra. The elements x_{ijk} can be held in a three-way array \mathbf{X} of size $I \times J \times K$ where I is

Table 1. Elemental analysis of humic substances.

Sample†	C	H	O	N	S	P	Al	Ca	Fe	Mn
g kg ⁻¹ dry matter										
EHa‡	581.3	36.8	340.8	41.4	4.4	2.4	< DL§	< DL	0.9	7×10^{-4}
EFa	501.2	42.8	426.1	37.5	8.9	1.2	< DL	< DL	0.1	< DL
WHa	547.2	40.4	385.4	14.7	3.6	3.1	< DL	3.1	0.9	< DL
WFa	536.3	42.4	418.1	10.7	2.9	1.2	< DL	6.2	1.7	< DL
LHa	638.1	37.0	312.7	12.3	7.6	< 0.1	-#	-	-	-
PHa	568.4	36.0	366.2	37.4	7.0	0.3	-	-	-	-

† Contents of C, H, O, N, S, and P are from the supplier's information. Contents of Al, Ca, Fe, and Mn were measured by ICP-AES on samples dissolved in NaOH solution.

‡ EFa, Elliott soil standard fulvic acid; EHa, Elliott soil standard humic acid; LHa, Leonardite standard humic acid; PHa, Pahokee peat reference humic acid; WFa, Waskish peat reference fulvic acid; WHa, Waskish peat reference humic acid.

§ Less than detection limit.

Not determined.

the number of samples, J the number of emission wavelengths and K the number of excitation wavelengths.

The PARAFAC modeling was conducted with MATLAB version 7.0.4, Release 14 (Mathworks, Natick, MA) using PLS_Toolbox version 3.5 (Eigenvector Research, Manson, WA). A non-negativity constraint was applied to each dimension to allow only chemically relevant results because negative concentrations and fluorescence intensities are chemically impossible. PARAFAC models with two to eight components were computed. The determination of the correct number of components in the data set was assessed by the core consistency diagnostic score, which should be close to 100% for appropriate models (Bro and Kiers, 2003).

FT-IR Analysis

The FT-IR spectra were obtained from KBr discs. Each disc contained about 1 mg of a solid humic substance sample and 80 mg KBr. The spectra were recorded in the 450 to 4000 cm^{-1} range on a PerkinElmer Spectrum One FT-IR Spectrophotometer (PerkinElmer Instruments, Norwalk, CT). Each sample was scanned 24 times with a resolution of 2 cm^{-1} . All spectra were normalized.

Solution ^{31}P NMR Analysis

For solution ^{31}P NMR analysis, the original IHSS samples (17–45 mg) were dissolved in 0.6 mL of 10 M NaOH, 1.0 mL of deionized water and 1.6 mL of D_2O , and were allowed to stand for 30 min with occasional vortexing. Samples were then centrifuged for 20 min at approximately 1500 $\times g$ to remove any undissolved material, which can increase line broadening. For these samples, almost all solid material was dissolved, thus little humic material was removed during centrifugation. The supernatants were then transferred to NMR tubes, and stored at 4°C before analysis within 24 h. Solution ^{31}P NMR spectra were acquired at 202.5 MHz on a Bruker AVANCE 500 MHz spectrometer equipped with a 10-mm broadband probe, using a 90° pulse, 0.68 s acquisition, 4.32 s pulse delay, 82.4 μsec dwell time, 17.9 μsec pulse width and 15 Hz spinning. The spectral width was 12135.9, and the number of points was 8192. The delay time was based on prior T1 experiments (Cade-Menun et al., 2002; McDowell et al., 2006). Temperature was regulated at 20°C (Cade-Menun et al., 2002). Total experiment time per sample was approximately 8 h (5500 scans). NUTS software (Acorn NMR, Livermore, CA, 2000) was used for peak analysis and spectral integration, based on total peak area (Cade-Menun, 2005), after standardizing the orthophosphate peak to 6 ppm. Spectra were processed with 15 Hz line broadening, using automated baseline-correction and peak-picking routines in the processing software. Peak assignments were based on previous work (Cade-Menun and Preston, 1996; Turner et al., 2003).

After NMR spectroscopy, a portion of the NMR sample was diluted ten-fold and analyzed element contents by ICP-OES (TJA IRS Advantage 1000 Radial ICAP spectrometer).

Enzymatic Hydrolysis

Humic substances, phytate (dodecasodium inositol hexaphosphate) and inorganic orthophosphate KH_2PO_4 (all at 0.322 mM P) were separately pre-incubated with the control, AlCl_3 or FeCl_3 (3.22 mM) in 100 mM acetate buffer (pH 5.0) at 22°C for 20 h. These mixtures were then equally divided into two aliquots. Water or *Aspergillus ficuum* 3-phytase (EC 3.1.3.8, purchased from Sigma-Aldrich, St. Louis, MO) solution (0.1 U mL^{-1}) was added into these aliquots. After a 20-h incubation interval at 37°C, soluble inorganic P (P_i) in these mixtures was determined as described below.

Separation of High- and Low-Molecular Weight Humic Substances

Humic substances (1 mL) in stock solutions were diluted by 50% and adjusted to pH 5.0 in 100 mM acetate buffer by mixing with 0.078 mL of 400 mM acetate buffer (pH 5.0), 0.068 mL of 2.5 M acetic acid, and 0.854 mL of water. The high- and low-molecular weight humic fractions were separated by filtering samples in Amicon Centricon centrifugal filter units (3000 molecular weight cutoff, Millipore Corp., Bedford, MA). The samples were filtered by spinning at 6500 $\times g$ at 4°C until most material had passed through. The filtrates were saved as low-molecular weight humic substances. The retentates were rinsed twice with total 1.5 mL of water and spun down to about 0.1 mL volume. The recovered retentates were brought up to the initial volume of 2 mL with 100 mM Acetate buffer (pH 5.0). The UV/visible absorbance of the two fractions at 260, 280, 400, and 600 nm was measured by an Agilent 8453 diode array UV/visible spectrophotometer (Agilent Technologies, Wilmington, DE). Fractions were diluted 1/10 to 1/200 by acetate buffer (100 mM, pH 5.0) to keep the absorbance readings within the linear regions of the instrument. The retentates and filtrates were analyzed for P_i and enzymatic hydrolyzable organic P, separately.

UV Irradiation

For UV irradiation treatments, 1.5 mL (10 mg P L^{-1}) of humic substances in 100 mM Acetate buffer (pH 5.0) was placed in a closed quartz cuvette. A Spectroline 11SC-1 short wave UV pencil lamp (254 nm, 4.5 mW cm^{-2} , Spectronics Corp., Westbury, NY) was placed near the cuvette (1 cm away) and the sample was irradiated for 4 h at 22°C. The irradiated samples were then subjected to enzymatic hydrolysis as described above.

Phosphorus Determination

Soluble inorganic orthophosphate (P_i) in the reaction mixtures was directly quantified by the molybdenum blue method (He and Honeycutt, 2005) modified from an earlier method (Dick and Tabatabai, 1977) by changing the measured wavelength to 850 nm and adding 2% sodium dodecyl sulfate. The P_i concentration in control samples was designated as inorganic P, while enzymatically hydrolyzable organic P was the increase of P_i concentration after 3-phytase incubation. Blank absorption caused by colored humic substances in an aliquot was measured without Reagent B (i.e., an ascorbic acid solution) as no absorption would yield from soluble orthophosphate without ascorbic acid (Negrin et al., 1995). A correction was made to account for the blank absorption by subtracting it from the absorption measured in the presence of Reagent B.

RESULTS AND DISCUSSION

Fluorescence Spectra of Humic Substances

PARAFAC models with two to eight components were computed for the humic substance data set. The core consistency diagnostic value for our data set was 99.1% for the three-component model and 15.3% for the four-component model, indicating that the three-component model was the most appropriate model for the six humic substances being investigated. The three-component PARAFAC model explained 99.7% of the variability in the data set.

The EM and EX spectra were estimated in the second and third loadings of the PARAFAC model, respectively. The EX spectra of Component 1 had a primary peak at

< 240 nm and a secondary peak at 318 nm and an EM spectra peak of 438 nm (data not shown). Component 2 had an EX peak of 252 nm and an emission peak > 498 nm. Component 3 had EX peak < 240 nm and a broad EM peak at 390 nm. Chen et al. (2003) have operationally delineating the EEM landscape into five regions corresponding to aromatic protein-like (two regions), fulvic acid-like, microbial byproduct-like, and humic acid-like organic matter materials. Using these delineations, Components 1 and 3 were classified as fulvic-acid like material and Component 2 was classified as humic acid-like material.

The concentrations of the components were estimated in the first loading of the PARAFAC model (Fig. 1). It is important to recognize that the relative distributions shown were based on their fluorescence signal contribution, rather than on a chemical concentration basis, which would require knowledge of their quantum fluorescence efficiencies, which were unknown. The two fulvic acid materials, EFa and WFa, both had the fulvic acid-like Component 1 accounting for 55% of distribution of the fluorescing components (Fig. 1). Similarly, three of the four humic acids (EHa, LHa, and PHa) had the humic acid-like Component 2 accounting for 56% of the component content. The Component 2 accounted for only 37% of the distribution for the WHa humic acid. Component one showed the greatest contrast between the two fulvic acids and the four humic acids, suggesting that this fluorophore could serve as a potential "marker" for fulvic acids. The substantial presence of each component in all the materials highlighted the continuum nature of NOM structure. Although humic and fulvic acids were operationally separated based on pH solubility, the resultant fractions shared similar components.

Infrared Characteristics of Humic Substances

FT-IR spectra of the six samples were typical for humic substances (Fig. 2). These FT-IR bands could be interpreted based on previous reports with humic substance

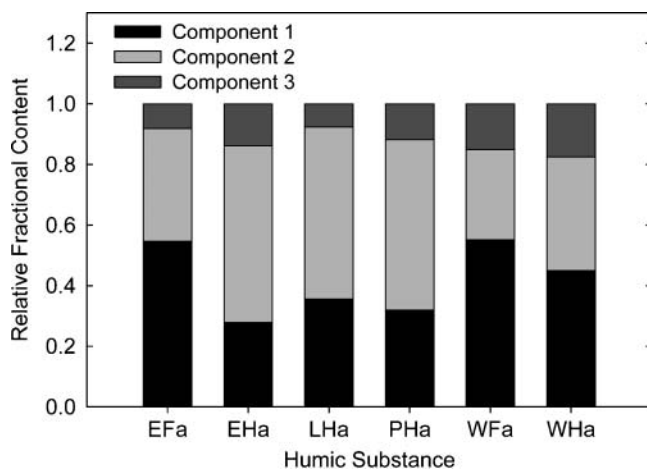


Fig. 1. Fluorescence characteristics of humic substances. Component 1 and 3 were contributed by fulvic acid-like organic matter materials, and component 2 humic acid-like organic matter materials. EFa, Elliott soil standard fulvic acid; EHa, Elliott soil standard humic acid; LHa, Leonardite standard humic acid; PHa, Pahokee peat reference humic acid; WFa, Waskish peat reference fulvic acid; WHa, Waskish peat reference humic acid.

(Agnelli et al., 2000; Francioso et al., 1998; Giusquiani et al., 1998; Olk et al., 2000; Tan, 2003). The broad band around 3400 cm^{-1} was assigned to O-H and N-H stretching, and the band at 2917 cm^{-1} or so to aliphatic C-H stretching. The band at 1720 to 1711 cm^{-1} was generally due to C=O stretching of COOH and other carbonyl groups. The sharp or shoulder band at 1627 – 1600 cm^{-1} was due to aromatic C=C vibrations, symmetric stretching of COO^- groups, and H-bonded C=O of conjugated ketones. The shoulder or weak bands at 1414 and 1367 cm^{-1} were preferentially assigned to aliphatic C-H, and O-H deformation, C-O stretching of phenolic OH, and COO^- antisymmetrical stretching. The broad band around 1217 cm^{-1} was attributed to C-O stretching and O-H deformation of carboxyls, phenols, and aromatic ethers. Unlike the other four spectra, a minor band at 1514 cm^{-1} was observed in the spectra of WFa and WHa, indicating the presence of ortho-substituted aromatic compounds (Francioso et al., 1998) in the two Waskish peat humic substances. The spectra of the four humic acids showed two absorbance bands at 1717 and 1613 with equal intensity. The spectra of the two fulvic acids showed a strong band at 1711 cm^{-1} and a weak band at 1613 cm^{-1} . These observations were consistent with the reported characteristics of Type I (humic substances) and Type II (fulvic acid) FT-IR spectra (Tan, 2003).

The assignment of the absorption band at 1100 to 1000 cm^{-1} was noteworthy. Bands in this region have been frequently assigned to alcoholic and polysaccharide C-O stretching or to vibrations of a SiO_2 -related impurity in humic substance (Agnelli et al., 2000; Giusquiani et al., 1998; Olk et al., 2000; Tan, 2003). Although they could not exclude that the band in this region may arise from C-O stretching or an inorganic impurity, Francioso et al. (1998) attributed it mainly to phosphate groups as a result of the high concentration of total P in their humic acids extracted by NaOH plus pyrophosphate. In the spectra of our six samples (Fig. 2), the intensity of this band changed from a minor band in WHa, EHa, WFa, and EFa, to a shoulder or very weak shoulder bands in PHa and LHa. This change followed the same decreasing order of P content in the six samples (Table 1), implying a correlation between the absorbance in this region and P content. Rulmont et al. (1991) reported that the common features of orthophosphates are the absence of bands in the 900 to 700 cm^{-1} region, and the strongest bands generally centered near 1050 cm^{-1} . The spectrum of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ showed a strong band 1012 cm^{-1} (Fig. 2). With these reference data in consideration, it was reasonable to assign the absorbance band at 1100 to 1000 cm^{-1} to phosphate groups in the humic substances. If rigorously confirmed with more samples, the characteristic P absorbance at 1100 to 1000 cm^{-1} would provide a simple and rapid diagnostic for P contained in humic substances.

Solution ^{31}P NMR Spectra of Humic Substances

Peak analysis and spectral integration by NUTS software showed that the major P species were orthophosphate and monoester in Elliott humic substances (Fig. 3). Orthophosphate diesters and phosphonates may

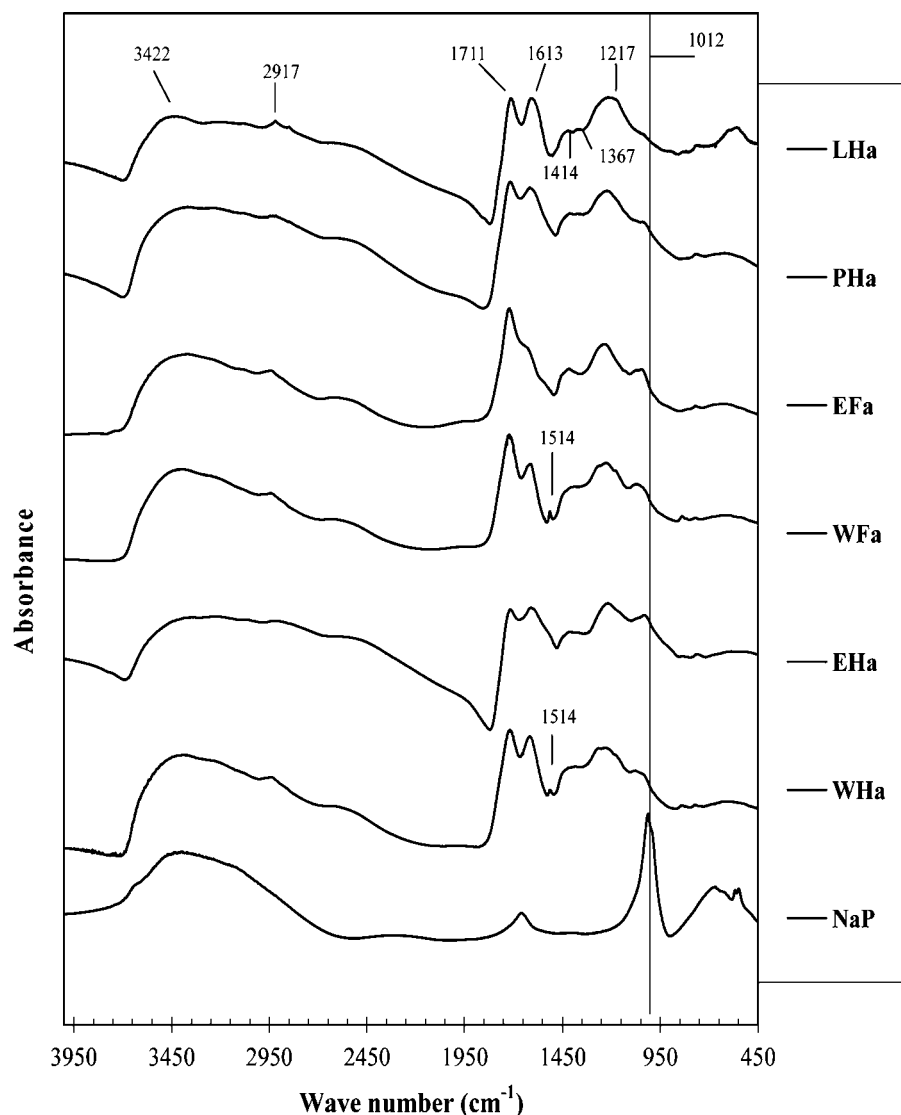


Fig. 2. FT-IR spectra of humic substances. NaP, trisodium phosphate dodecahydrate; WHa, Waskish peat reference humic acid; EHa, Elliott soil standard humic acid; WFa, Waskish peat reference fulvic acid; EFa, Elliott soil standard fulvic acid; PHa, Pahokee peat reference humic acid; LHa, Leonardite standard humic acid.

be present, especially for the EHa spectrum, but these cannot be reliably distinguished from the background noise. Thus, we calculated the relative percentages of only orthophosphate and monoesters in the humic substances. In EHa, we found that 21% of the P was orthophosphate and 79% orthophosphate monoesters; in EFa, 40% of the P was orthophosphate and 60% orthophosphate monoesters. Only orthophosphate was detected in WHa and WFa spectra, which were from a peat source. All these P forms have been found in other humic and fulvic acids reported previously (Bedrock et al., 1995; Mahieu et al., 2000; Maier et al., 1989). Unlike our data, however, Bedrock et al. (1995) found that most of the P in their peat humic substances was organic.

Soluble Inorganic Phosphorus and the Effects of Aluminum and Iron Ions on its Solubility

Near stoichiometric recovery of inorganic P was observed after 20-h control incubation (Fig. 4). Ferric

ions precipitated 60% of soluble inorganic P while Al ions precipitated only 5% of soluble inorganic P. Less than 1% of the P in phytate was detected in the absence of the enzyme, indicating the stability (i.e., little spontaneous hydrolysis) of phytate during the incubation period. About 24% of P in EHa and EFa was soluble P_i . The additives reduced the detectable P_i in EHa and EFa to 17%. Although ^{31}P NMR analysis indicated that all P in both WHa and WFa was inorganic orthophosphate, the two humic substances contained little soluble P_i (6 and 4% of total P, respectively). It seemed that organic matter source (soil vs. peat) was a more important influence than organic matter fraction (humic acid vs. fulvic acid) on the properties of P in these humic substances. Elemental analysis (Table 1) showed that EHa and EFa did not contain detectable levels of Ca; however, the molar Ca/P ratio was 0.8 for WHa, and 4 for WFa. Ferric and Al ions did not significantly influence the soluble P_i in Waskish peat humic substances (Fig. 4). Thus, we consider that Ca- P_i -humic matter complexes constituted the major P form in the Was-

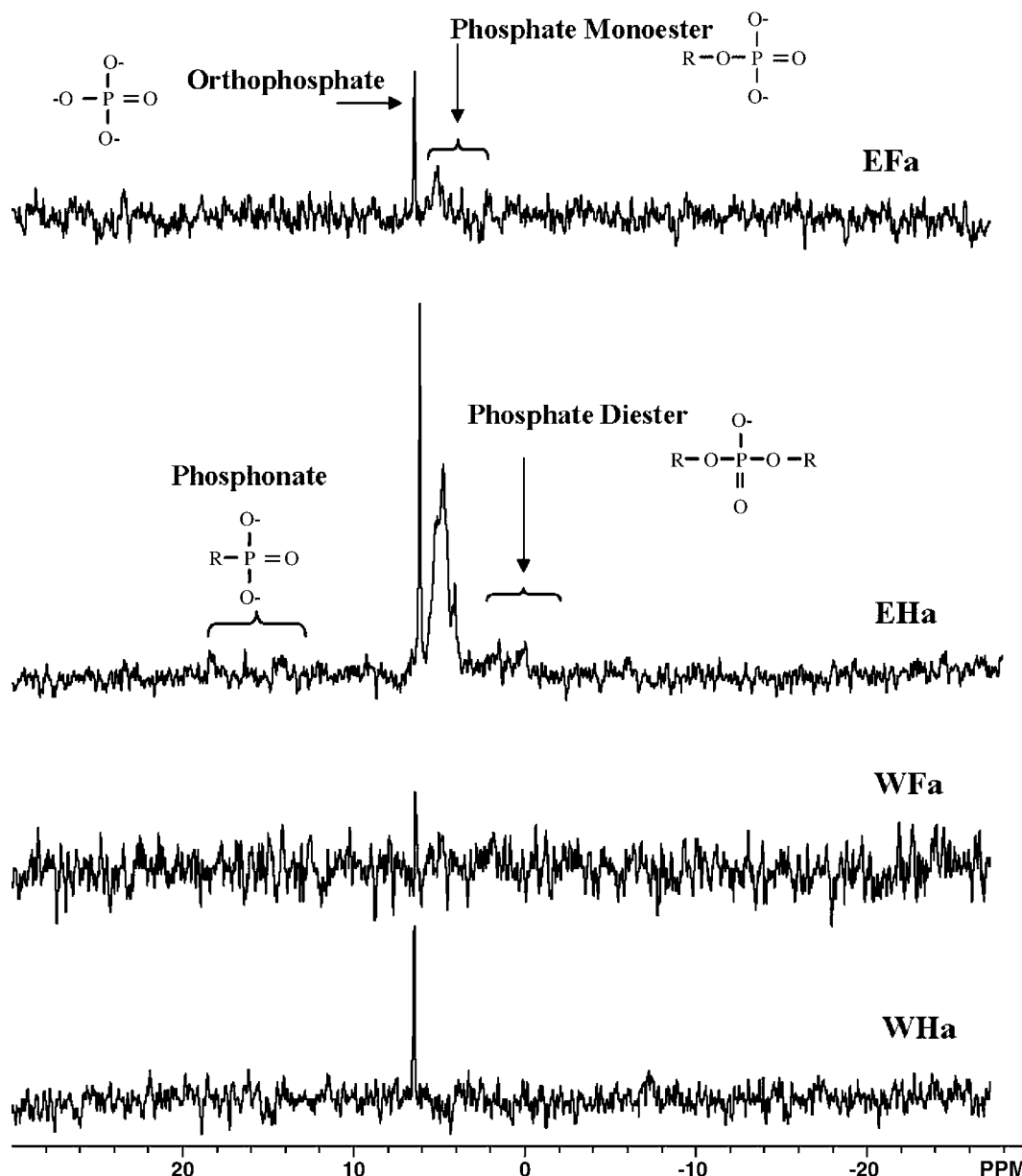


Fig. 3. Solution ^{31}P NMR spectra of humic substances. Spectra are plotted with 15 Hz line-broadening. WHa, Waskish peat reference humic acid; WFa, Waskish peat reference fulvic acid; EHa, Elliott soil standard humic acid; EFa, Elliott soil standard fulvic acid.

kish peat humic and fulvic acids as P has been found in both mobile and recalcitrant Ca humates (Mahieu et al., 2000).

The presence of soluble P_i in these humic substances was surprising because the procedure used to obtain humic substances would have removed soluble P_i from humic substances (IHSS, 2005). Our observation, however, seemed to be supported by previous reports (Hens and Merckx, 2001; Hino, 1989; Pant et al., 1994b) that soluble P_i (or molybdenum reactive P) occurred in fractions associated with a wide molecular-weight range of organic compounds when soil solutions were subjected to gel filtration. This soluble P_i might be associated with the core structures of organic matter through weak van der Waals interactions (Celi and Barberis, 2004) or metal-organic matter complexes (Hens and Merckx, 2001), which could be partially hydrolyzed under acid conditions (Gerke, 1992; Gerke and Jungk, 1991). Thus, this portion

of P in humic substances would be considered the most labile P and potentially bioavailable for plant uptake.

The Effects of Aluminum and Iron on the Release of Enzymatically Hydrolyzable Organic Phosphorus

When 3-phytase was included in the reaction mixture, 94% of P in phytate was released as soluble P_i , indicating the effectiveness of the enzyme on releasing orthophosphate from organo-phosphorus compounds (Fig. 5). Enzymatic incubation released an additional 16% (EHa) or 18% (EFa) of P from Elliott soil humic substances. Substrate specificity research has shown that 3-phytase is able to hydrolyze a variety of phospho-monoesters as well as phytate (Hayes et al., 2000; He and Honeycutt, 2001). Thus, the amounts of soluble P_i

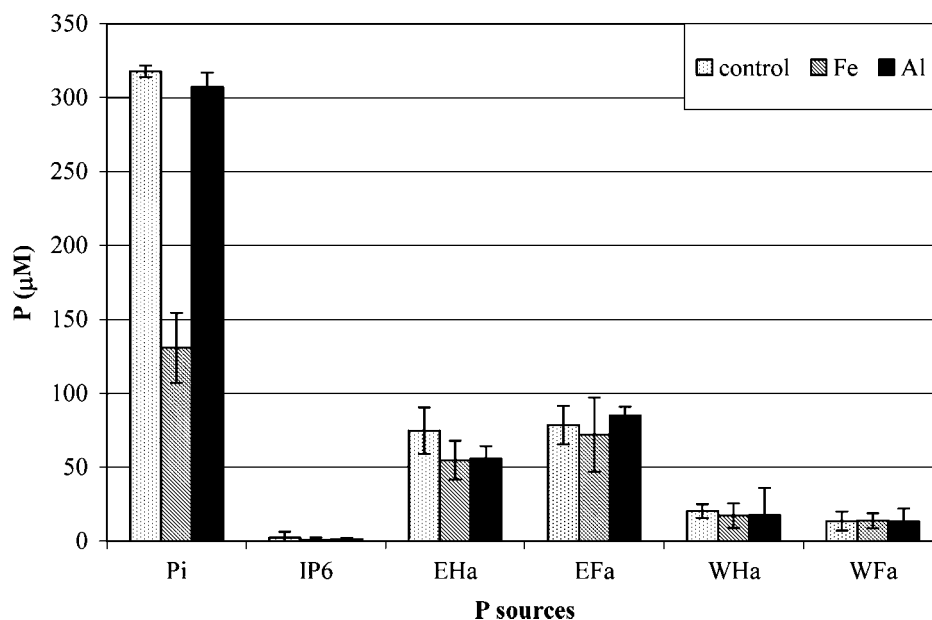


Fig. 4. Soluble P_i in reaction mixtures in the absence and presence of Fe or Al ions (3.22 mM each). Each reaction mixture contains 0.322 mM P. Pi, KH_2PO_4 ; IP6, phytate; EHa, Elliott soil standard humic acid; EFa, Elliott soil standard fulvic acid; WHa, Waskish peat reference humic acid; WFa, Waskish peat reference fulvic acid. Values are averages \pm standard deviations ($n = 6$).

increased due to the enzymatic hydrolysis should be considered general monoester P including phytate. Comparing with the ^{31}P NMR data, lower concentrations of phosphomonoesters were detected in EHa and EFa by the enzymatic hydrolysis, which seemed reasonable because the latter method would detect only the hydrolyzable portion of phosphomonoesters. Only 4% (WHa) to 2% (WFa) of additional P associated with Waskish peat humic substances was released by 3-phytase hydrolysis. The results were roughly in agreement with the ^{31}P NMR data. It was also possible that the low signal/noise ratios in the ^{31}P NMR analysis might have ham-

pered the detection of the minor amounts of monoester P in these samples, or the interferences in the colorimetric determination may have overestimated the P concentration after enzymatic hydrolysis.

No increase in soluble P_i was observed in the reaction mixture of phytate plus Fe or Al. This observation was consistent with previous reports (Dao, 2003; He et al., 2006). The concentrations of soluble P_i were much greater in the reaction mixtures of KH_2PO_4 with Fe (63 μM P) or Al (267 μM P) than in the reaction mixtures of phytate with Fe (1 μM P) or Al (1 μM P) (Fig. 5), indicating that the two metals affected the

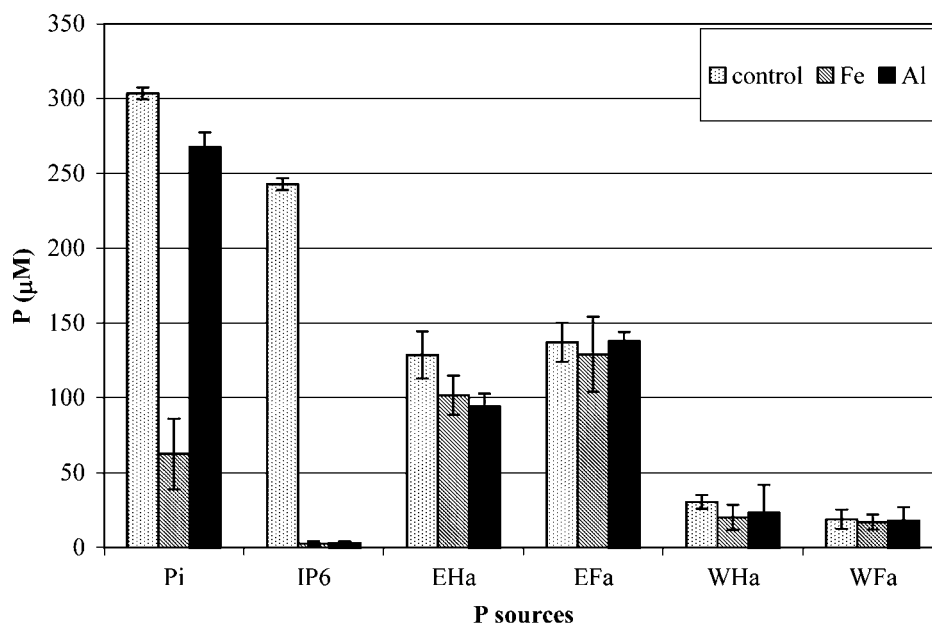


Fig. 5. Increase of soluble P_i by 3-phytase hydrolysis. Reaction mixtures contain 0.322 mM P and none (control) or 3.22 mM Fe or Al ions. Pi, KH_2PO_4 ; IP6, phytate; EHa, Elliott soil standard humic acid; EFa, Elliott soil standard fulvic acid; WHa, Waskish peat reference humic acid; WFa, Waskish peat reference fulvic acid. Values are averages \pm standard deviations ($n = 6$).

release of P in phytate by forming insoluble metal-phytate complexes, rather than precipitating soluble P_i after it released from phytate by 3-phytase. Unlike phytate, the inhibitory influence of metal additives on enzymatic release of humic- or fulvic-associated P was negligible. These results suggested that these two metals may have preferential interactions with functional groups other than phosphate esters in humic and fulvic acids (Levesque and Schnitzer, 1967; Lobartini et al., 1998).

Phosphorus Distribution in Low- and High-Molecular Weight Fractions of Elliott Soil Humic Substances

Because of their relatively large concentrations of reactive hydrolyzable organic P, EHa and EFa were separated into low- and high-molecular weight fractions with a molecular weight cutoff of 3000. The UV/visible absorbances at selected wavelengths are listed in Table 2. Absorbances at A_{260} and A_{280} have been used to monitor the levels of organic compounds eluted from gel filtration chromatography of NOM (Hens and Merckx, 2001; Hino, 1989; Pant et al., 1994a, 1994b). Absorbances at A_{400} and A_{600} and their ratios are useful to characterize humic and fulvic acids (Tan, 2003). Based on the relative intensity of the absorbances at A_{260} and A_{280} , we concluded that most organic compounds in EHa possessed molecular weights > 3000 . The ratio of low- and high-molecular weight organic compounds in EFa was about 1:2. The ratios of A_{400} and A_{600} were 15 and 3.6 for the low- and high-molecular weight fractions of EHa, and 22 and 10 for corresponding fractions of EFa. These data were consistent with characteristics of typical humic substances; humic acids contain more high-molecular weight compounds and have lower ratios of A_{400} and A_{600} than fulvic acids (Tan, 2003).

Unlike large portions of organic compounds in high-molecular weight fractions, nearly 90% of soluble P_i was in the low-molecular weight fractions of EHa and EFa before enzymatic hydrolysis (Fig. 6). These observations further suggested that soluble P_i detected in the EHa and EFa solutions arose from P species that were weakly associated with organic matter in humic substances. This might have become ionic orthophosphate (P_i) under the experimental conditions, thus partitioning to the lower-

molecular weight fractions detectable by the molybdenum blue method. Incubation with 3-phytase increased soluble P_i detected in both low- and high-molecular weight fractions of these two humic substances. The increases were 55 μM (i.e., $\mu mole P L^{-1}$ humic solution) (17% of total P in the humic solution before partitioning) and 46 μM (14% of total P) for the low- and high-molecular fractions of EHa, respectively, and 33 μM (10% of total P) and 26 μM (8% of total P) for the low- and high-molecular fractions, respectively, of EFa. These data suggested that the enzymatically hydrolyzable organic P was about equally distributed in these two fractions.

Ultraviolet Irradiation of Humic Substances

After the four humic substances were irradiated for 4 h by UV light, soluble P_i concentrations were 123 μM for EHa, 114 μM for EFa, 49 μM for WHa, and 31 μM for WFa (Fig. 7). Subtracting soluble P_i in non-irradiated humic substances (Fig. 4), UV irradiation released an additional 20% of the P in EHa, 11% of the P in EFa, 9% of the P in WHa, and 5% of the P in WFa. Similar to non-irradiated materials, enzyme hydrolysis increased P_i most in Elliott soil humic substances. The increase (29%) in P_i by enzymatic hydrolysis in irradiated EHa nearly doubled the increase in P_i after hydrolysis for non-irradiated EHa (16%). However, UV irradiation seemed not to facilitate the hydrolysis of organic P in EFa because the 16% increase in irradiated EFa was equivalent to the 18% increase in P_i for non-irradiated EFa after enzymatic hydrolysis. UV irradiation did not increase enzymatic release of organic P in Waskish peat humic substances. The portion of unaccountable P could have resided in other forms of organic P and monoester P that were protected from 3-phytase attack by organic moieties of humic substances, and orthophosphate-metal-humic substance complexes. The latter would be the major form in WHa and WFa. Further characterization by reducing (He et al., 2006) and/or chelating agents (Dao, 2004) and other orthophosphate-releasing enzymes (He and Honeycutt, 2001; He et al., 2004) may reveal the potential of hydrolysis of those unaccountable P species.

The presence of ultraviolet-sensitive humic-phosphorus complexes has been reported previously (Francko and Heath, 1979, 1982). These studies have demonstrated that the high-molecular weight P fraction of an acid bog lake sample did not release a detectable amount of soluble P_i in the presence of acid molybdate reagents or on alkaline phosphatase hydrolysis, but did release P_i when briefly exposed to low intensities of UV light. This humic-phosphorus complex might be considered as orthophosphate sorbed to ferric-dissolved humic substances and released by UV-induced photoreduction of ferric iron to the ferrous state (Francko and Heath, 1979; 1982). The fact that UV irradiation did not facilitate enzymatic hydrolysis of EFa, WHa, and WFa was consistent with these previous observations, suggesting the presence of P_i -humic complexes in the four humic substances. UV irradiation can also abiotically degrade humic substances to relatively simple compounds (Chen et al., 1978; De Haan, 1993). It is reasonable to assume

Table 2. UV/visible absorbance of low- (LMW) and high- (HMW) molecular weight fractions of Elliott humic acid (EHa) and fulvic acid (EFa) †.

	A_{260}	A_{280}	A_{400}	A_{600}
EHa				
LMW	$10.5 \pm 0.3^\ddagger$	8.9 ± 0.3	1.8 ± 0.1	0.12 ± 0.00
HMW	184 ± 10	163 ± 9	62.0 ± 4.0	17.2 ± 2.6
EFa				
LMW	39.4 ± 1.0	31.0 ± 1.0	4.4 ± 0.0	0.20 ± 0.01
HMW	73.0 ± 10	61.5 ± 8.5	11.7 ± 1.3	1.15 ± 0.15

† The original concentration before filter fractionation was 0.322 m mole $P L^{-1}$ solution in acetate buffer (100 mM, pH 5.0) in both acids, equivalent to 4.2 mg EHa mL^{-1} solution or 8.3 mg EFa mL^{-1} solution. Fractions were diluted 1/10 to 1/200 in acetate buffer (100 mM, pH 5.0) to keep the absorbance readings within the linear regions of the instrument. Total absorbance in undiluted fractions were obtained by multiplying the dilution factors.

‡ Average \pm standard deviation ($n = 2$).

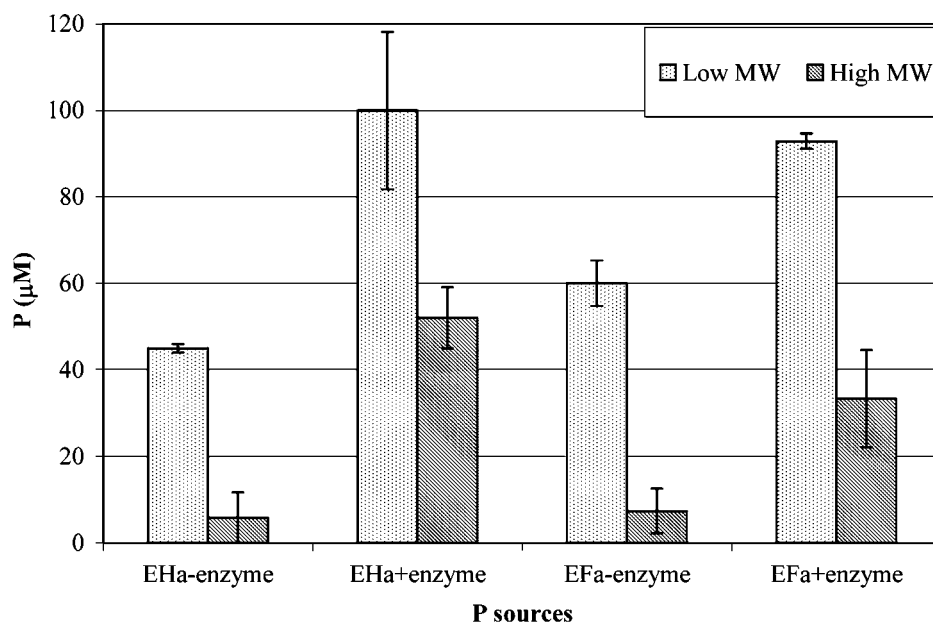


Fig. 6. Soluble P_i in reaction mixtures in the absence and presence of 3-phytase in low- and high-molecular weight fractions of Elliott soil humic substances. EHa, Elliott soil standard humic acid; EFa, Elliott soil standard fulvic acid. Values are averages \pm standard deviations ($n = 3$).

that organic P associated with these simple compounds would be more enzymatically hydrolyzable. This was the likely mechanism through which UV irradiation increased enzymatically hydrolyzable P in EHa.

CONCLUSIONS

Fluorescence and FT-IR spectra demonstrated that EHa, WHa, EFa, and WFa were typical humic and fulvic acids. The presence of ortho-substituted aromatic compounds, however, was more evident in WHa and WFa. Our observations indicated that various forms of phosphates with different degrees of hydrolysis were present

in humic substances, and source (soil vs. peat) was a more important factor than fraction (humic acid vs. fulvic acid) in influencing the forms and lability of P in these humic substances. The fact that a lesser percentage of labile P in Waskish peat humic substances than in Elliot soil humic substances could be explained by the lack of organic P and the presence of ortho-substituted aromatic compounds in the former, which suggested that chemical composition of humic substances played a critical role in regulating P availability associated with humic substances. This work demonstrated that biotic and abiotic releases of P in a humic substance and effects by environmental factors (such as metal ions) could be

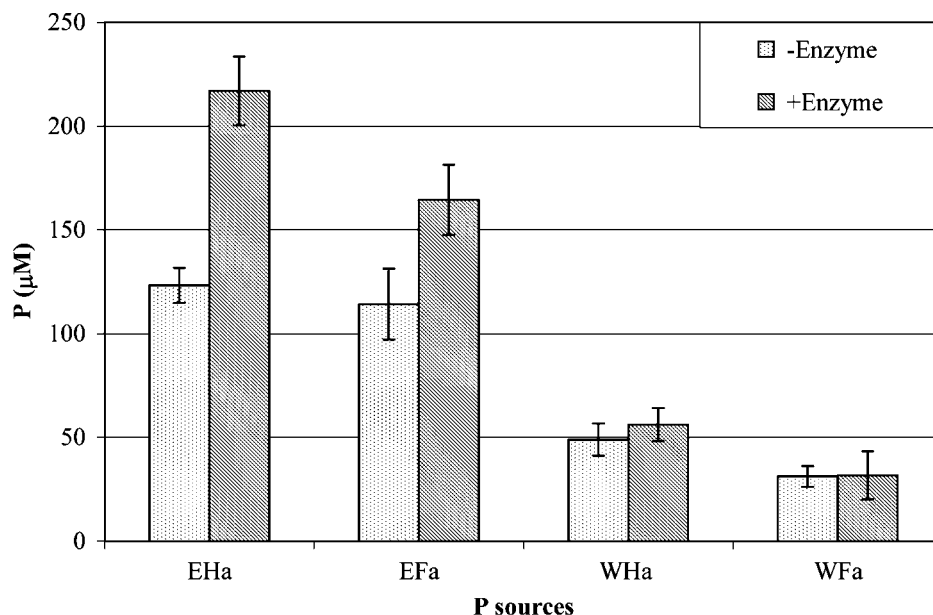


Fig. 7. Soluble P_i in reaction mixtures in the absence and presence of 3-phytase soil. Before the enzymatic incubation, the humic substance solutions were irradiated by UV light for 4 h. EHa, Elliott soil standard humic acid; EFa, Elliott soil standard fulvic acid. Values are averages \pm standard deviations ($n = 4$).

investigated by enzymatic hydrolysis and UV irradiation, respectively. Application of this method in future research may lead to improved understanding of the dynamics and mechanisms of transformations of natural organic matter-bound P from unavailable to bioavailable forms in the environment.

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